REMARKS

Applicants respectfully request reconsideration of this application in view of the above amendment and the following remarks.

Applicants have amended Claims 1, 8, 76, and 83. Applicants have cancelled Claims 11, and 86. Applicants have added new Claim 139.

Claims 1 and 76 have been amended to include electroporation as the delivery method of introducing a plasmid vector into the muscle cells of the female animal, and the plasmid encodes a growth hormone releasing hormone or its respective analog. Support for this amendment can be found in the Specification at paragraphs [0015] and [0020], as well as in the original disclosure of cancelled Claims 2, 3, 77, and 78.

Pending in the application are Claims 1, 5-10, 12-13, 76, 80-85, 87-88, and 137-139.

I. Information Disclosure Statement:

The Examiner has not considered U.S. Patent No.: 5,134,210, reference 10, on the IDS submitted by the Applicants because the author listed on the IDS was incorrect.

Applicants respectfully submit that the typographical error of the last two numbers of the patent caused this confusion. The correct U.S. Patent is 5,134,120, and not 5,134,210. Applicants have attached a copy of the correct patent reference and request an Examiner's Amendment to correct the IDS of Reference 10 to be U.S. Patent No.: 5,134,120, titled "Use of Growth Hormone to Enhance Porcine Weight Gain," with Boyd et al., listed as inventors.

II. Rejections Under 35 U.S.C. §112 First Paragraph.

The Examiner has maintained the rejections of Claims 1, 5-13, 76, 80-88, 137 and 138 under 35 U.S.C. §112 First Paragraph. The Examiner is of the opinion that the specification is enabling for a very narrow claim, which Applicants have now presented as new Claim 139, the Examiner is of opinion that the specification does not reasonably provide enablement for enhancing growth in an offspring from ANY female mammal using a vector expressing ANY GHRH or analog thereof during gestation.

The Examiner has cited that the interpretation of enablement was considered in view of the Wands factors (MPEP 2164.01(a)). As such, the Examiner is of the opinion that analysis of Wands factors combined with several cited references demonstrate that the working embodiments of these broad aspects are not reasonably predictable, as such, it would require "undue" experimentation for the Artisan to find the working embodiments and therefore, the claims are not enabled for their fully claimed scope, an some claims are not enabled whatsoever.

The Examiner has presented at least three specific arguments for rejecting the claims under 35 U.S.C. §112 First Paragraph, represented below as (a), (b) and (c).

(a) The Examiner is of the opinion that the route of administration of vector using the term "parenteral" is too broad because "intravenous or intraperitoneal" methods would be considered parenteral and were not taught in the specification.

In response to Examiner's objection to the broad term "parenteral" routes of administration of vector, Applicants have amended independent Claims 1 and 76 to be limited to:

"electroporating into muscle cells of the female mammal an effective amount of a vector"

Additionally, Claims 11 and 83 that indicate other routes of introducing a vector have been canceled. Thus, Applicants submit that the above amendments and cancellation of claims render moot the Examiner's argument in connection with the term "parenteral."

(b) The Examiner is of the opinion that the claims broadly encompass the use of ANY "growth hormone releasing hormone or analog thereof," and such broad language includes the other hormones, such as ghrelin. Furthermore, the Examiner is of the opinion that "No guidance was provided as to whether ghrelin expression resulted in an offspring from a female mammal that has improved or enhanced growth."

Applicants respectfully submit that "[C]laims nee not be limited to exemplification or preferred embodiments in order to satisfy enablement requirements." *Ex parte Gould*, 6 U.S.P.Q.2d 1680 (B.P.A.I. 1987). "Enablement is a legal determination of whether a patent enables one skilled in the art to make and use the claimed invention. It is not precluded even if some experimentation is necessary, although the amount of experimentation needed must not be unduly extensive. . . . Furthermore, a patent needs not teach, and preferably omits, what is well know in the art." *Hybritech Inc. v. Monoclonal Antibodies, Inc.*, 231 U.S. P.Q. 81 (Fed. Cir. 1986).

Applicants respectfully submit that the terms "growth hormone," "growth hormone releasing hormone," and "growth hormone releasing hormone analog," are common meanings of the terms, as evidenced by the many citations in the specification. More specifically, Applicants provided references that indicated the terminology and relative skill of one having ordinary skill in the art. These references provide an abundance of guidance on both synthetic and naturally occurring GHRH and GHRH analogs, as evidenced in paragraph [0011]. Paragraph [0011] is presented below to emphasize that sufficient guidance was present in Applicants' specification. As such, Applicants respectfully submit that any experimentation needed to practice the claimed invention is not an undue experimentation.

[0011] There are issued patents which address administering novel GHRH analog proteins (U.S. Pat. Nos. 5,847,066; 5,846,936; 5,792,747; 5,776,901; 5,696,089; 5,486,505; 5,137,872; 5,084,442; 5,036,045; 5,023,322; 4,839,344; 4,410,512; RE33,699) or synthetic or naturally occurring peptide fragments of GHRH (U.S. Pat. Nos. 4,833,166; 4,228,158; 4,228,156; 4,226,857; 4,224,316; 4,223,021; 4,223,020; 4,223,019) for the purpose of increasing release of growth hormone. A GHRH analog containing the following mutations has been reported (U.S. Patent No. 5,846,936): Tyr at position 1 to His; Ala at position 2 to Val, Leu, or others; Asn at position 8 to Gln, Ser, or Thr; Gly at position 15 to Ala or Leu; Met at position 27 to Nle or Leu; and Ser at position 28 to Asn. The GHRH analog which is the subject of U.S. Patent Application Serial No. 60/145,624, herein incorporated by reference, does not contain all of the amino acid substitutions reported in U.S. Patent No. 5,846,936 to be necessary for activity. The invention of U.S. Patent Application Serial No. 60/145,624 differs from U.S. Patent No. 5,756,264 in two respects. First, the invention of U.S. Patent Application Serial No. 60/145,624 concerns an analog of growth hormone releasing hormone which differs from the wild type form with significant modifications which improve its function as a GH secretagogue: decreased susceptibility to proteases and increased stability, which would prolong the ability to effect a therapy, and increased biological activity, which would enhance the ability to effect a therapy. The analog of U.S. Patent Application Serial No. 60/145,624 lacks the substitution at position 8 to Gln, Ser, or Thr present in the GHRG analog of U.S. Patent No. 5,756,264. In addition, in one aspect of the invention of U.S. Patent Application Serial No. 60/145,624, the invention utilizes a DNA encoding the GHRH analog linked to a unique synthetic promoter, termed SPc5-12 (Li et al., 1999), which contains a proximal serum response element (SRE) from skeletal a-actin, multiple MEF-2 sites, MEF-1 sites, and TEF-1 binding sites, and greatly exceeds the transcriptional potencies of natural myogenic

promoters. The uniqueness of such a synthetic promoter is a significant improvement over, for instance, issued patents concerning a myogenic promoter and its use (e.g. U.S. Pat. No. 5,374,544) or systems for myogenic expression of a nucleic acid sequence (e.g. U.S. Pat. No. 5,298,422).

Applicants further submit that one of ordinary skill in the art would NOT have isolated paragraph [0051] and read it in a vacuum without consideration of the immediate previous paragraphs or out of context with the entire rest of the specification that uses the term GHRH and GHRH analog, as defined in dozens of cited references. One of ordinary skill in the art would not confuse GHRH or GHRH analogs with ghrelin, as indicated by the Examiner. Paragraph [0049] through [0052] read as follows:

[0049] The term "growth deficiencies" as used herein is defined as any health status, medical condition or disease in which growth is less than normal. The deficiency could be the result of an aberration directly affecting a growth hormone pathway (such as the GHRH-GH-IGF-I axis), indirectly affecting a growth hormone pathway, or not affecting a growth hormone pathway at all.

[0050] The term "growth hormone" as used herein is defined as a hormone which relates to growth and acts as a chemical messenger to exert its action on a target cell.

[0051] The term "growth hormone releasing hormone" as used herein is defined as a hormone which facilitates or stimulates release of growth hormone.

*[0052] The term "growth hormone releasing hormone analog" as used herein is defined as a protein which contains amino acid mutations and/or deletions in the naturally occurring form of the amino acid sequence (with no synthetic dextro or cyclic amino acids), but not naturally occurring in the GHRH molecule, yet still retains its function to **enhance synthesis and secretion of growth hormone**.(Emphasis Added)*

Applicants submit that one of ordinary skill in the art would have known that "ghrelin" enhances **secretion** of growth hormone, but it **DOES NOT** enhance **synthesis**, as such, ghrelin would be excluded from the definition of a growth hormone releasing hormone analog, as defined in paragraph [0052]. In fact, Applicants submit that the Anderson citation (Anderson et al., 2004 Exp. Biol., Med 229:291-302), which was cited by the Examiner, clearly distinguishes this difference between GHRH and ghrelin on page 292 paragraph (a) Growth Hormone Releasing Factor (GHRH); and paragraph (c) Ghrelin.

Applicants strongly disagree with the Examiner's assertion that "no guidance" was provided in connection with the use of "ghrelin." In fact, Applicants' specification in paragraph [0171] defined "ghrelin" as "an endogenous ligand" of the growth hormone secretagogue receptor, and NOT as a growth hormone releasing hormone or a GHRH analog, as shown below:

> *[0171] In another embodiment of the present invention, ligands for the growth hormone secretagogue receptor (GHS-R) give a similar result as delivery of a GHRH nucleic acid. A skilled artisan is aware of the many different GHS-R ligand structural types known in the art, all of which work through the GHS-R. Examples include MK-0677 from Merck (Whitehouse Station, NJ), GHRP-6 (for review see Bowers, 1998) and* ***ghrelin, an endogenous ligand*** *(Kojima et al., 1999; Dieguez and Casanueva, 2000). Others include hexarelin (Europeptides), L-692,943 (Merck & Co.; Whitehouse Station, NJ), NN703 (Novo Nordisk; Bagsvaerd, Denmark) or any compound which acts as an agonist on the GHS-R receptor, all of which are well known to a skilled artisan (see, for example, Pong et al. (1996); Howard et al. (1996); or Smith et al. (1997)).*(Emphasis Added)

Furthermore, the use of such a GHS-R ligand in combination with a GHRH nucleic acid would have an added enhancement, as shown below in paragraph [0172]:

[0172] A skilled artisan is aware that the GHS-R is upstream of GHRH and increases GHRH release from the pituitary gland. In a specific embodiment a GHS-R ligand is given orally (such as by adding to the feed or drinking water), which would amplify the effects of GHRH on causing release of GH from the pituitary gland. ***In this embodiment, the GHRH nucleic acid delivery of the present invention would get an added enhancement.***. (Emphasis Added)

The Court has held:

"It is the function of the specification, not the claims, to set forth the "practical limits of operation" of an invention. One does not look to the claims to find out how to practice the invention they define, but to the specification. The specification as a whole must be considered in determining whether the scope of enablement provided by the specification is commensurate with the scope of the claims." *In re Johnson and Farnham,* 558, F2.d 1008, 194 U.S.PQ 187, 195 (C.C.P.A 1977).

The Examiner is of the opinion that only SEQID No. 1 and SEQID NO.: 8 are the only enabled embodiments for GHRH or GHRH analogs.

Applicants respectfully disagree.

First, the Court has held that: "[S]ection 112 of 35 U.S.C. does not permit an Examiner to study a disclosure, to formulate a conclusion as to what he (the examiner) regards as the broadest invention supported by the disclosure, and then to determine that the claims are broader than the Examiner's conception of what 'the inventions' is." *In re Borkowski and Van Venrooy*, 164 U.S.P.Q. 6412 (C.C.P.A. 1970).

Applicants respectfully submit that GHRH was first isolated in humans from a pancreatic tumor producing GHRH (see Guillemin, R. Growth hormone-releasing factor from a human pancreatic tumor that caused acromegaly, Science, 1982, volume 218, pages 585-587, referenced in paragraph [0071] of Applicants' specification), and not from its natural hypothalamic source. In this particular case, the patient was first diagnosed with acromegaly (hyperproduction of growth hormone), and then the tumor source of stimulation was identified. One of ordinary skill in the art would have known, for over 20 years, that GHRH could be delivered "parenterally" and still have physiological effects, as long as the GHRH or GHRH analog thereof can gain access to the subjects general circulation. One of ordinary skill in the art would also have been aware of the many different GHRH analogs that effect the GHRH-GH growth axis, as Applicants have specifically addressed with listed references for many such GHRH analogs (See paragraphs [0010]-[0014], and [0069]-[0071]). Those specific references together with the defined terms of GHRH analogs described in [0051]-[0052], give extensive guidance to the artesian of ordinary skill in the art.

Applicants submit that the cited examples of GHRH analogs and the definition in paragraph [0052] of a GHRH analog is sufficient to set forth the "practical limits of operation" while providing protection against future patentable inventions related to electroporating a nucleic acid expression vector encoding a GHRH or GHRH analog into the muscle cells of a pregnant mother to enhance the growth of an offspring.

Additionally, an entire sections (paragraphs [0106] - [0110]) of the specification were devoted to site directed mutagenesis for the purpose of showing how GHRH analogs can be made. The specification clearly states the GHRH analogs in the context of a distinct amino acid sequence.

(c) The Examiner is of the opinion that the claims broadly encompass ANY female animal, while the specification teaches the effects of GHRH and HV-GHRH only on pigs and rats. Furthermore, the Examiner cited a 19 year old paper (Hammer et al., 1986), which indicate that transgenic mice and transgenic pigs expressing human growth hormone (hGH) respond differently. Thus, the

Examiner is of the opinion that Hammer's teaching indicates not all mammals "predictably" respond to expression of heterologous proteins.

Applicants submit that the Hammer et al, 1986 paper **SHOULD NOT** be used to make a comparison between the current invention because Applicants are not using transgenic animals. One of ordinary skill in the art would know that transgenic animals are animals from a specific genetic lineage, whereas Applicants' invention can be practiced on any genetic lineage.

For example, transgenic animals, such as described in the Hammer 1986 paper, result from the introduction of a purified double-stranded DNA sequence into the chromosomes of the fertilized mammalian egg. If this transferred genetic material (i.e., transgene) is integrated into one of the embryonic chromosomes, the animal will be born with a copy of this new information on the same chromosome in every single cell of the mature animal. The foreign DNA must integrate into the host genome prior to the doubling of genetic material that precedes the first cleavage or a mosaic animal may be produced in which many cells do not possess the new gene. For this reason, the transgene DNA is introduced into the zygote at the earliest possible stage, i.e., the pronuclear period immediately following fertilization. If the germ cells of the founder (mosaic or not) transmit the transgene stably, then all descendants of this animal are members of a unique transgenic lineage. In contrast, Applicants' method does not introduce vector DNA into the chromosomes and the method can be practiced on a fully mature mammal.

One of ordinary skill in the art knows that integration of foreign DNA into the embryonic genome generally is a random event with respect to the chromosomal locus. Therefore, the probability of identical integration events in two embryos of the same species receiving the same transgene is **overwhelmingly unlikely**, and even more unlikely in two entirely different species, such as described in the Hammer 1986 paper. In addition, it is almost impossible to regulate exactly how many copies of the transgene will be introduced into the embryo and how many will join together to integrate (usually at a single site) as a single linear array called a concatamer. Animals that are positive for the transgene are bred into specific animal lineages and used for experimentation. Thus, without specific breeding strategies, two differently injected zygotes will likely express the transgene differently depending on which chromosome the foreign DNA was integrated, which is why the phenotype for different transgenic linages are varied. This can be especially true for different transgenic animals produced in different species. Applicants submit that transgenic animals are tools for experimentation and not practical solutions for solving problems.

In contrast, Applicants' invention introduces a plasmid expression vector into the muscle cells of any mature mammals (See for example Claim 1). All working examples use "regular," not transgenic, animals. As such, the expression gene of interest is NOT present in every cell of the treated animals body from birth, as is the case for transgenic animals. Thus, Applicants submit that comparing of transgenic animals with animals of the current invention as suggested by the Examiner is scientifically flawed. Because transgenic animals were are not even suggested as part of the Applicants' invention, the Hammer 1986 paper cannot be considered as relevant.

The Examiner is further of the opinion that the effects of GHRH and HV-GHRH of only female rats and female pigs have been taught in the specification and other female animals would not react similarly to the therapy and "undue" experimentation would be needed for each and every other mammalian species.

Applicants submit that Example 20 and Example 21 of the specification indicate that an expression plasmid that encodes for either native GHRH or a GHRH analog was administered to either pregnant rats or sows during the latter part of gestation. As a result, perinatal morbidity and mortality of the offspring in both species were reduced, their growth was enhanced, and this response was sustained into postnatal life (rats: Khan AS et al., Endocrinology. 2002 Sep;143(9):3561-7; pigs: Khan AS, et al., Am J Physiol Endocrinol Metab. 2003 Jul;285(1):E224-31). This phenomenon occured despite the markedly disparate placental structures of these two species; in the pig, six membranes separate the fetal and the maternal circulations, whereas there are only three in the rat, and pointing one more time to the fact that this is a reproducible cross-species phenomenon. To confirm these studies the Applicants and others have varied conditions and species. Numerous parties have since used the invention described in the application and found the results reproducible both for the large animal species described herein (i.e. pigs), but maintained into completely different conditions (farm conditions rather than laboratory conditions), and for other animal species (i.e. beef cattle and dairy cattle, maintained in farm conditions). These findings were described in Brown et al., "Immune Enhancing Effects of Growth Hormone Releasing Hormone Delivered by Plasmid Injection and Electroporatoin," Molecular Therapy Vol 10(4) p 644-651, which has been attached as Exhibit A

Applicants submit that while animal species react heterogeneously in some circumstances, some basic mechanisms are similar or identical, and the GHRH-GH axis appears to be one of these basic mechanisms. In **all mammalian species GHRH stimulates GH** (and the literature is more than abundant on this point, including the Anderson et al., 2004 paper cited by the Examiner). Applicants' invention demonstrates that GHRH or analogs thereof can be delivered to an animal muscle using a nucleic acid expression vector instead of a recombinant protein. Furthermore, Applicants have provided working and reproducible examples of this method in different mammalian species. In fact, Applicants' specification shows that the species can be as different as rats and pigs, and the method has the same beneficial effects.

III. Response to Arguments.

The Examiner is of the opinion that delivering plasmid DNA to a mother using a parenteral route, which includes administration of the plasmid DNA intravenously or intraperitoneally constitutes "*in utero* gene therapy." The Examiner is also of the opinion that the parenteral route of Claim 8 encompasses the issue of enablement addressed by Aanjani and Anderson pages 19-20.

In response, Claims, including Claim 8, using the parenteral routes of delivery have been cancelled.

Under the Examiner's broad definition of "*in utero,*" an expecting mother getting a flu shot would be considered getting an *in utero* therapy, because her getting a flu shot would either directly or indirectly benefit the fetus. That cannot be right. The dictionary definition of "*in utero*" is "**within** the uterus [Emphasis added]." *See*, page 209 of "A Dictionary of Genetics," Sixth Ed., by Robert C. King and William D. Stansfield, Oxford University Press, 2002, pertinent pages are attached as Exhibit B. For an *in utero* therapy to occur, the agent must be given directly to penetrate the uterus and arrive at the fetus or, in the alternative, the agent must cross the placenta barrier.

Applicants submit that "*in utero* gene therapy" does not occur when the plasmid vector is electroporated into the muscle cells of the female mammal prior to or during gestation of the offspring. Firstly, the DNA vector plasmids in this invention are NOT given directly to penetrate the uterus to arrive at the fetus. Secondly, Applicants submit that one of ordinary skill in the art knows that DNA vector plasmids are too big to cross the placenta even if they somehow managed to get into the general circulation. As shown in a paper published by Khan AS, et al., Am J Physiol Endocrinol Metab. 2003 Jul;285(1):E224-31, which was published after the filing date of the application, a very sensitive PCR reaction failed to produce any evidence of plasmid in the placenta, amniotic fluid, or the fetus of the animals that had a plasmid introduced into muscle cells, as described in Applicants' application.

The current claims, as amended, **do not, and cannot,** encompass "*in utero* gene therapy." Plasmids are not being delivered to the fetus. Rather, plasmids are delivered to muscles of the female mammal whose expression of the delivered nucleic material improves the growth and health of the offspring.

IV. Response to Arguments.

Additionally, the Examiner has re-stated that one of the problems with gene therapy is that an artisan cannot predict the effects of an expressed transgene, as evidenced by the Hammer 1986 paper. Furthermore, the Examiner is of the opinion that the Applicants have not provided any guidance with respect to predictably using GHRH or GHRH analogs in all species of mammals.

As stated above, Applicants submit that the animals of the current invention cannot be compared to the transgenic models of the Hammer 1986 paper (See Above). Furthermore, Applicants are not claiming ANY or all transgenes. Applicants are claiming a method of using mammalian GHRH or analog thereof, which encoded on a DNA vector, as commonly defined and recognized by one of ordinary skill in the art. Because GHRH has been extensively characterized both at the nucleic acid level and hormone level, those with ordinary skill in the art recognize and could reproduce all methods. Guidance has been provided in the way of abundant citations and working examples. Cross species use of GHRH has already been discussed above.

Applicants submit that Example 14, paragraphs [0146-0147], show a direct working embodiment of the Applicants' invention using a single pregnant pig. Furthermore, Example 20, paragraphs [0174]-[0196], reproduces these results in 6 different pregnant pigs and compares the results with 6 control pigs. A total of 132 piglets confirmed that the Applicants' method of improving or enhancing growth in an offspring from a female mammal was significantly reproducible.

Applicants further showed in Example 21, paragraphs [0197]-[0210], that the same method could be used reproducibly in a completely different species of mammal, a rat. These plasmids were delivered to muscle cells of the animals by electroporation and showed that expression was statistically reproducible in at least in two separate species. Applicants submit that NONE of the reference cited by the Examiner indicate that the instant method is related to previously failed gene therapy experiments. Furthermore, the Examiner's skepticism of the Applicants' method does not undermine the evidence presented in the working examples of the Applicants specification.

The Court has held that:

"[S]ection 112 of 35 U.S.C. does not permit an Examiner to study a disclosure, to formulate a conclusion as to what he (the examiner) regards as the broadest invention supported by the disclosure, and then to determine that the claims are broader than the Examiner's conception of what 'the inventions' is." *In re Borkowski and Van Venrooy*, 164 U.S.P.Q. 6412 (C.C.P.A. 1970).

The Examiner CANNOT use personal skepticism and "what if" situations that are based upon failed transgenic mouse experiments to reject claims based upon working, statistically significant, and reproducible examples, as described in Applicants' specification.

Further, "[I]t is **NOT** a function of a claim to specifically exclude either possible inoperative substances or ineffective reactant proportions." [Emphasis added] *In re Dinh-Ngnven and Strenhagen,* 492 F. 2d 851, 181 U.S. P.Q 46 (C.C.P.A. 1974).

Applicants respectfully assert that the claims as amended are fully enabled and that the effectiveness and utility of the claimed methods are established in the Specification and are not at issue. As noted above, Applicants have amended the claims to clarify that the vector is electroporated into muscle cells of the female mammal.

VIII. Conclusions

Applicants respectfully submit that, in light of the foregoing Amendments and comments, Claims 1, 5-10, 12-13, 76, 80-85, 87-88, and 137-139 are in condition for allowance. A Notice of Allowance is therefore requested.

If the Examiner has any other matters which pertain to this Application, the Examiner is encouraged to contact the undersigned to resolve these matters by Examiner's Amendment where possible.

Respectfully submitted,

T. Q Chwang

T. Ling Chwang
Registration No. 33,590
JACKSON WALKER L.L.P.
2435 North Central Expressway, #600
Richardson, TX 75080
Tel: (972) 744-2919
Fax: (972) 238-3319

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EXHIBIT A

Immune-Enhancing Effects of Growth Hormone-Releasing Hormone Delivered by Plasmid Injection and Electroporation

Patricia A. Brown,[1] William C. Davis,[2] and Ruxandra Draghia-Akli[1,*]

[1]*ADViSYS, Inc., 2700 Research Forest Drive, Suite 180, The Woodlands, TX 77381, USA*
[2]*Department of Veterinary Microbiology and Pathology, CVM, Washington State University, Pullman, WA 99164-7040, USA*

**To whom correspondence and reprint requests should be addressed. Fax: (281) 296 7333. E-mail: ruxandradraghia@advisys.net.*

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Growth hormone-releasing hormone (GHRH) is a hypothalamic hormone with both direct and indirect functions in the maintenance of immune status under physiological and pathological conditions. In this study, 52 Holstein heifers were evaluated for the effects of a plasmid-mediated GHRH treatment on their immune function and on the morbidity and mortality of treated animals. In the third trimester of pregnancy, 32 heifers received 2.5 mg of a myogenic GHRH-expressing plasmid by intramuscular injection followed by electroporation, while 20 heifers were used as controls. No adverse effects were associated with either the plasmid delivery or GHRH expression. At 18 days after plasmid administration, GHRH-treated animals had increased numbers of $CD2^+$ $\alpha\beta$ T-cells ($P < 0.004$), $CD25^+CD4^+$ cells ($P < 0.0007$), and $CD4^+CD45R^+$ cells ($P < 0.016$) compared to controls. These increases were maintained long term after treatment and correlated with plasmid expression. At 300 days post-GHRH therapy, $CD45R^+/CD45R0^-$ naïve lymphocytes were significantly increased in frequency ($P < 0.05$). Natural killer lymphocytes ($CD3^-CD2^+$) were also increased. As a consequence of improved health status, body condition scores of treated animals improved (3.55 vs. 3.35, $P < 0.0001$). Hoof pathology was also reduced with treatment. The mortality of heifers was decreased (3% vs. 20% in controls, $P < 0.003$). Collectively, these results indicate that the myogenic GHRH plasmid can be successfully electrotransferred into a 500-kg mammal and expressed for prolonged periods of time, ensuring physiological levels of GHRH. The plasmid injection followed by electroporation could prove an efficient method for the systemic production of therapeutic proteins and may provide a useful means for basic research in relevant animal models.

Key Words: electroporation, GHRH, GH, IGF-I, immunity, plasmid

INTRODUCTION

Traditionally, plasmid-mediated gene transfer technology has been limited in scope because expression levels following naked DNA transfer have been low, only a fraction of that achieved after viral-mediated gene transfer [1]. Furthermore, for large mammals the doses of plasmid needed to achieve sufficient levels of circulating proteins, other than for vaccination purposes, are sometimes unrealistic as a common therapy or for basic research purposes. Conversely, results obtained in rodents are not always translated successfully to larger mammals or humans [2]. Physiologically relevant levels of plasmid DNA transfer and transgene expression can be accomplished by utilizing the electroporation technique [3–5]. Electroporation has proven useful *in vivo* in patients on whom drug delivery to malignant tumors has been performed [6]. Many recent studies of plasmid delivery enhanced with electroporation have used skeletal muscle cells as an ideal target for direct plasmid transfer [7,8] and have shown that the injected muscle can be used as a bioreactor for the persistent production and secretion of proteins at physiological levels into the bloodstream.

Our previous studies showed that delivery of a plasmid that expresses growth hormone-releasing hormone (GHRH) by intramuscular injection followed by electroporation is scalable and represents a promising approach for long-term stable production of secreted proteins in pigs and dogs [9,10].

Two hypothalamic hormones, GHRH and somatostatin, regulate the secretion of growth hormone (GH); GHRH stimulates GH release from the pituitary, and somatostatin inhibits its release, resulting in the pulsa-

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tile release of GH into the circulation. Studies in different animal models and humans have shown that GHRH has many physiological roles. Among these is a stimulatory effect on immunity, both through stimulation of the GH/insulin-like growth factor-I (IGF-I) axis and directly as an immune modulator [11,12]. Some experiments have suggested that GHRH can also modulate immune functions through brain mechanisms that are also involved in the regulation of sleep [13].

Although the cumulative observations clearly show that significant progress has been made in establishing a role for GHRH in development and regulation of the immune system, detail is still lacking on how GHRH mediates those effects. Until now, there has been no dynamic large animal model in which the biological effects of GHRH could be monitored over periods of time sufficient to measure changes in immune function and general improvement in health.

We propose that our model of delivering the hormone by plasmid injection followed by electroporation can be extended to the study of the underlying immune changes that may occur with GHRH administration. Moreover, the direct effects of GHRH plasmid administration on morbidity and mortality rates in the treated herds can be investigated. An additional advancement that has increased the potential of using large animals for GHRH studies has been the development of strong expression vectors that can be delivered at relatively low quantities (2.5 mg in animals with average weight of more than 500 kg) into muscle cells. The present study was undertaken to demonstrate the potential of the plasmid-electroporation model and answer two questions: (1) Does a plasmid-mediated GHRH therapy produce long-term beneficial effects on the immune function in cattle? (2) Are these changes clinically relevant? The results of our study demonstrate that plasmid-mediated GHRH treatment stimulates an increase in T-cell and natural killer cells in peripheral blood that appears to be associated clinically with an improvement in immune function. The clinical consequences are reduced morbidity and mortality and improved body condition scores in the treated heifers compared to controls.

RESULTS

Biochemistry and CBC Values

At 300 days posttreatment, the total white blood cell counts were similar between groups. Nevertheless, the percentage of circulating lymphocytes was increased in GHRH-treated animals (47.4 ± 3.3% vs. controls, 37.8 ± 5.3%, $P < 0.06$). We also observed a physiological increase in hemoglobin (11.55 ± 0.15 g/dl in GHRH-treated vs. 10.9 ± 0.15 g/dl in controls, $P < 0.02$) and red blood cells (7.65 ± 0.1 million/ml vs. 7.3 ± 0.2 million/ml, $P < 0.07$) at this time point. We found no differences between the groups in other CBC or serum biochemistry panels at any time point tested. These were within the normal range of values for cattle. Glucose and insulin levels were not different between groups (Table 1).

TABLE 1: Glucose and insulin levels in GHRH-treated animals and controls

	GHRH-treated	Control	*P* value
Glucose, mg/dl			
60 DIM	43.9 ± 1.4	41.8 ± 4.5	0.67
100 DIM	72.2 ± 1.4	69.3 ± 3.8	0.5
Insulin, μU/ml			
60 DIM	11.6 ± 2.5	9.4 ± 3.2	0.59
100 DIM	7.8 ± 1.5	7.4 ± 0.6	0.8

Glucose and insulin levels were measured at 60 and 100 DIM. Values are presented as means ± SEM.

Immune Markers

The total number of white blood cells, differentials, and flow cytometric (FC) profiles were similar between groups at day 0. At 18 days posttreatment, $CD2^+$ values were increased in treated animals by 14%, but there was no change in controls compared to baseline values: 43.4 ± 1.7% vs. 37.9 ± 1.4%, $P < 0.004$, in GHRH-treated cattle and 37.3 ± 2.1% vs. 38.8 ± 1.8% in controls (Fig. 1A). The $CD4^+/CD8^+$ ratio increased in treated animals (day 18–day 0 = 8 ± 0.6%, $P < 0.04$), mostly due to an increase in $CD4^+$ cells (29.1 ± 0.7% at day 18 vs. 24.5 ± 0.8% at day 0). During the same period $CD4^+CD45R^+$ naïve lymphocytes increased by 53% with the GHRH treatment: day 18, 11.1 ± 0.4% vs. day 0, 7.4 ± 0.4% in GHRH-treated animals, $P < 0.016$, and day 18, 8 ± 0.7% vs. day 0, 7.2 ± 0.8% in control animals (Fig. 1B). $CD25^+CD4^+$ cells were also significantly increased with treatment: day 18, 4.3 ± 0.3% vs. day 0, 1 ± 0.1 in GHRH-treated animals, $P < 0.001$, and day 18, 3.8 ± 0.2% vs. day 0, 1.7 ± 0.2 in controls.

At 300 days posttreatment, when a more comprehensive panel was performed, we found that $CD45R^+/CD45R0^-$ naïve lymphocytes were significantly more numerous in treated animals (0.98 ± 0.08) than in controls (0.91 ± 0.08), $P < 0.05$ (Fig. 1C). $CD2^+CD3^+\gamma\delta^-$ cells were more numerous with treatment: 68.5 ± 1.4% of all $CD2^+$ cells vs. 60 ± 5.6% in controls, $P < 0.02$.

Mortality in Treated Animals

The mortality of the heifers (involuntary cull rate) was different between GHRH-treated animals and controls. During the 360-day study, none of the treated heifers died, while 20% of control heifers had to be culled ($P < 0.003$). The causes of death were the following: one Johne's disease, one systemic infection from hoof conditions and an infected cut, one animal with severe hoof problems complicated by rear leg paralysis, and one severe mastitis case. One treated animal was culled

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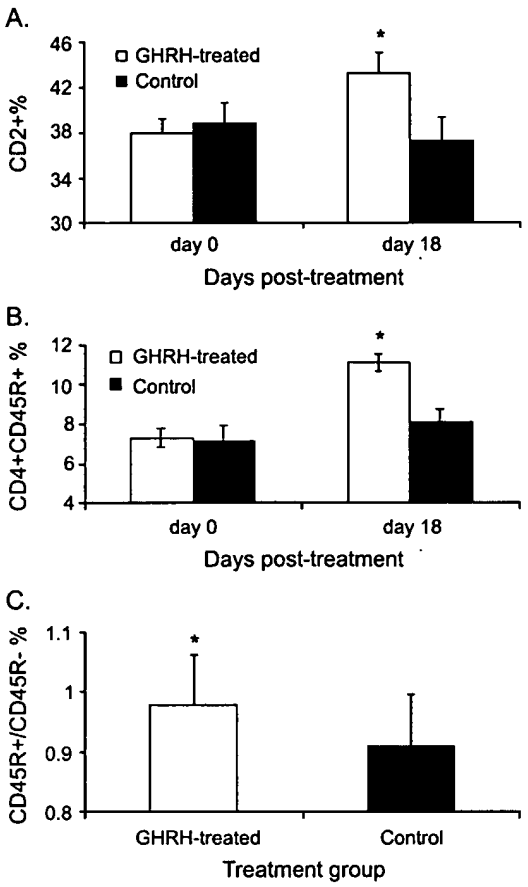

FIG. 1. Percentage of (A) $CD2^+$ cells and (B) $CD4^+CD45R^+$ naïve T-cells at days 0 and 18 after treatment and of (C) the ratio of $CD45R^+/CD45R^-$ naïve T-cells at 300 days posttreatment. Values are presented as means ± SEM, $^*P < 0.001$.

due to an accident. The overall involuntary cull rate prior to 120 days in milk production (DIM) was decreased by 40% with the treatment.

Body Weights and Body Condition Score

Body condition scores (BCS) of heifers differed between groups at the time of stress and negative energy balance, at 60 to 80 DIM. Heifers treated with pSP-HV-GHRH showed an improvement ($P < 0.0001$, Fig. 2) in BCS between 60 and 80 DIM. During the first 100 DIM, treated animals lost an average of 3.5 kg (0.06% of total body weight) ($P < 0.02$, Table 2) while control cows lost on average of 26.4 kg (4.6% of body weight at 60 DIM). The better BCS correlated with an increase in the serum IGF-I levels: day 100–day 60 = 22.4 ± 4 ng/ml for GHRH-treated heifers (119.7 ± 6.9 ng/ml at day 100 vs. 97.3 ± 6.6 ng/ml at day 60) vs. 8 ± 7.4 ng/ml for controls (99.8 ± 3.9 ng/ml at day 100 vs. 91.8 ± 6.8 ng/ml at day 60) ($P < 0.04$).

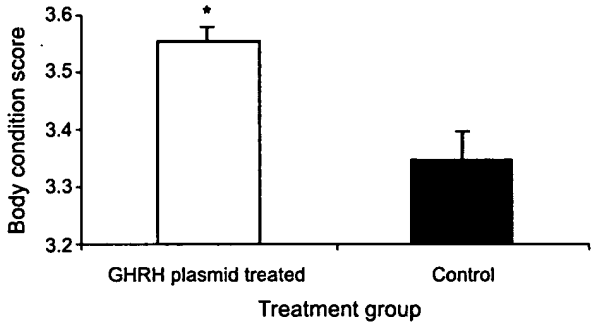

FIG. 2. Body condition scores in heifers treated with pSP-HV-GHRH versus controls at 60 to 80 DIM. Body condition scores differed between treatment groups, $P < 0.0001$.

Morbidity

The herd had significant hoof pathology at the beginning of the study prior to plasmid administration. Foot problems, most probably of bacterial origin [14], were also one of the principal causes of morbidity in these animals throughout the study. The proportion of animals that had worsening foot problems throughout the course of the study was 40% higher for controls compared to the treated animals: 7 of 32 GHRH-treated animals versus 7 of 20 controls. The overall hoof score improvement did not reach statistical significance ($P < 0.4$) due to high interanimal variability in the control group.

DISCUSSION

Recently, significant progress has been made to enhance plasmid delivery *in vivo* and subsequently to achieve physiological levels of a secreted protein. Intramuscular injection of plasmid followed by electroporation has been used successfully in ruminants for

TABLE 2: Weight of heifers 60 days precalving (DPC) and 100 and 135 DIM, weight difference between these time points, and percentage difference compared to baseline weight

	GHRH-treated	Control	*P* value
Weight (kg)			
60 DPC (A)	540 ± 28.1	543 ± 42.7	0.38
100 DIM (B)	536.4 ± 32.9	514.2 ± 48	**0.04**
135 DIM (C)	544.9 ± 33.1	533.5 ± 44.8	0.17
Difference (kg)			
A-B	−3.5	−26.4	**0.02**
A-C	4	−9.1	0.1
Difference (%)			
A-B/A	−0.5%	−4.6%	**0.02**
A-C/A	0.1%	−1.4%	0.1

Values are presented as means ± SEM. *P* value for each specific comparison between treatments is given.

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vaccination purposes [15,16]. We have demonstrated previously in mice, rats, pigs, and dogs that GHRH can be efficiently produced by muscle cells and released into the circulation using this technology. Here, we apply this method to a cattle model to study the immune-enhancing effects of GHRH, and we show that the plasmid injection/electroporation is scalable and provides the framework for potential human applications of this technology [17]. Furthermore, we have followed expression for more than 300 days after a single administration of GHRH plasmid, demonstrating the long-term applicability of this treatment system.

Stimulation of the GH axis has been shown to have a variety of positive immunostimulatory effects [18]. A substantial body of research exists that supports the production of GHRH, GH, and IGF-I by cells of the immune system [19], suggesting that immune function might be regulated by both autocrine and paracrine mechanisms. It has also been suggested that the increased morbidity in the elderly may be causally related to two changes that occur with aging, decreased GH/IGF-I production and decreased immune function [20].

Studies have shown that administration of GHRH or its analogs in the elderly resulted in profound immunoenhancing effects, both short- and long-term after therapy, with increased numbers of lymphocytes, monocytes, B-cells and cells expressing T-cell receptor $\alpha\beta$, and T-cell receptor $\gamma\delta$ [21].

GH secretagogues have been shown to inhibit the initiation of tumors in aged mice [22] in parallel with increased thymic cellularity. Debilitated dogs with cancer that received plasmid-mediated GHRH had improved immune parameters and red blood cell production [23]; the improved quality of life of the dogs was maintained for at least 1 year after a single plasmid administration [24]. Furthermore, overexpression of heterologous or homologous GH in transgenic mice led to significant stimulation of some parameters of immune function, such as increases in the absolute weight of the thymus and the spleen and in the mitogenic responses of splenocytes to concanavalin A, lipopolysaccharide, and phytohemagglutinin [12].

IGF-I modulates immune function and has two major effects on B-cell development, with potentiation and maturation, and acts as a cofactor for B-cell proliferation. The recombinant protein increases the number of pre-B- and mature B-cells in bone marrow [25]. The mature B-cell remains sensitive to IGF-I [26].

It has been generally assumed that plasmids that contain CpG motifs are potent agents for inducing inflammatory cytokines *in vivo* that, in turn, promote activation of transgene product-specific B- and T-cells [27]. The plasmid pSP-HV-GHRH contains a total of 183 CpG sequences. Of these, 93 are part of a class shown to be nonimmunostimulatory or even to suppress the effects of immunostimulatory CpGs (so-called CpG-N motifs, including CCG, CGG, and CGCG sequences as reviewed in [28]). The remaining 90 CpGs are consistent with immune-stimulatory sequences (CpG-S motifs). The specific effect, if any, of these CpG motifs on the biological activity of plasmid is unknown and will require further investigation. While control animals did not receive empty plasmid, it is unlikely that the described immune stimulation may have been nonspecific and mediated through the CpG motifs present in the plasmid backbone, considering the long-term hormonal and body composition changes in the GHRH-treated animals.

This study demonstrated a correlation between the improved health status of GHRH-treated heifers and changes in the composition of peripheral blood mononuclear cells (PBMC) over the time investigated. Differences were noted in the frequency of $CD2^+$ T-cells, the $CD4^+/CD8^+$ ratio, and the frequency of $CD45R^+$ naïve T-cells. The clinical consequences of enhanced immune function were reduced morbidity and mortality in GHRH-treated cows. Further studies are needed to determine if improvement in immune function is the result of direct interaction of GH or IGF-I with T-cells (mediated through GHRH) or the result of indirect effects resulting from an interaction with monocytes and antigen-presenting cells [29]. GH appears to affect cell function by promoting the survival of progenitor cells in the thymus and also T-cell function in peripheral lymphoid tissue. GH and IGF-I have been reported to increase T-cell function *in vitro*. Data are limited, especially in large animals such as cattle. Our finding of an increase in $CD2^+$ T-cells, possibly associated with the increase in naïve T-cells, is consistent with an effect on the thymus and an increase in output of naïve $CD4^+$ ($CD45R^+$) T-cells. It is known that T-cell activation depends in part on the number of T cell receptors (TCR), which is further dependent on small co-stimulatory molecules, such as CD2. CD2 can significantly reduce the threshold of triggered TCRs required for cell proliferation and cytokine synthesis. As a consequence, CD2 stimulates T-cell activation in the absence of direct engagement of antigen-specific TCR and increases expression of the TCR ζ chain, resulting in enhanced CD3 receptor density [30], and cytokine production [31]. Enhanced TCR and CD3 constitutive expression was reported to be a factor in innate immunity or autoimmunity [32]. Further studies are now needed to determine if the subtle changes shown in our study are associated with changes in the capacity of naïve T-cells to respond to pathogenic organisms. The potential that needs to be explored is whether these types of effects could also influence maturation of the immune system in the fetus and result in enhanced survival as noted in these studies.

Methods that could decrease the rate of animal loss under farm conditions, improve BCS, and reduce pathology in the herd are highly desirable. A current approach used to increase herd productivity is admin-

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istration of bovine somatotrophin (bST). However, bST administration to cows does not affect the animal's body weight, BCS, and energy balance at conception and during pregnancy [33,34] and may have detrimental effects in some cases. Some undesirable side effects of GH protein therapies may be due to the fact that treatment with recombinant exogenous GH protein delivers only one GH isoform, abolishing the natural episodic pulses of GH, and inducing supraphysiological levels of GH. By contrast, no side effects have been reported for recombinant GHRH therapies. We confirmed this finding long term with glucose and insulin levels, which can be abnormal during GH therapies, but were normal throughout this study.

Although recombinant GHRH protein therapy stimulates normal cyclical GH secretion with virtually no side effects, the short half-life of GHRH *in vivo* requires frequent administration [35,36], and the therapy is expensive and clinically impractical. A plasmid-mediated method could overcome this limitation to GHRH use. Because of the physiological feedback regulation of the GHRH–GH–IGF-I axis in normal mammals, a wide range of plasmid doses may be efficacious.

In the present study, GHRH-treated dairy cattle had decreased morbidity and mortality rates. Culling (mortality) is a major economic problem in the farm animal industry. There are many reasons for culling animals, separated into involuntary (persistent hoof problems, persistent mastitis, nonbreeders, disease, or death) and voluntary culling (breeding stock or lower producing animals). Due to the high percentage of involuntary culling, voluntary cull decisions revolving around rational economic parameters (e.g., maintenance of herd size) are typically held to a minimum. The average overall cull rate in North America is approximately 36% [37]. In our case, the cull rate was 20% for controls, while only 1 of the 32 GHRH-treated animals was culled, due to an accident.

The digital dermatitis lesions that constituted the major hoof pathology were attenuated by the GHRH treatment. Studies have shown that as much as 29% of dairy cattle and 4% of beef cattle have gross lesions of digital dermatitis and that spirochetes are involved in more than 60% of the cases [38]. The immune response to the spirochetes is of short duration [39], thus, to diminish the infection burden, a stable long-term therapy would be preferable.

Previous work showed that changes in serum IGF-I postpartum may help predict both nutritional and reproductive status in dairy cattle [40]. Also, it has been shown that concentrations of IGF-I in serum are associated with BCS [41]. This association was confirmed in our study, with IGF-I levels that were maintained in treated animals throughout stress periods and correlated to increased immune surveillance and body mass.

Body condition scores and maintenance of body weight are indicators of adequate nutrition, breeding ability, and recognition of health status in dairy herds [42–44]. Body condition is a reflection of the body fat reserves carried by the animal [45,46]. These reserves can be used by the cow during early lactation, when the animals tend to be in a negative energy balance, with a loss of body condition and decrease in body weight [42,47]. In early lactation, body condition scores between 3.4 and 3.7 are considered in the optimum range. Minimized BCS loss translates to decreased mobilization of body tissue, resulting in better health status and productivity. As shown in the present experiment, between 60 and 80 DIM, control animals showed a significant decrease in total body weight and BCS, while treated cows maintained their initial weight and BCS, which may explain their reduced morbidity and mortality.

In this study, decreased morbidity and culling rates, increased BCS, and positive changes in the immune system have been achieved in a large animal with a single plasmid-mediated GHRH treatment. These clinical effects

TABLE 3: Monoclonal antibodies used to specify peripheral blood mononuclear cells

mAb	Ig isotype	Specificity
MUC2A [52]	IgG2a	CD2 expressed on αβ T-cells, a subset of γδ T-cells, and a $CD2^+CD3^-$ population of lymphocytes containing NK cells
BAT76 [52]	IgG2a	CD2 as noted above
MM1A [53]	IgG1	CD3 T-cell receptor expressed on αβ and γδ T-cells
ILA-11A [54]	IgG2a	CD4 expressed on T-helper/inducer T-cells
CACT138A [52]	IgG1	CD4 as noted above
CACT187A[a]	IgG1	CD4 as noted above
B29A [55]	IgG2a	CD5 expressed on αβ and γδ T-cells and a subset of B-cells
7C2B[a]	IgG2a	CD8α expressed on cytotoxic/immunomodulatory T-cells
CACT80C [52]	IgG1	CD8α as noted above
BAT82A [52]	IgG1	CD8β as noted above
CACT116A [56]	IgG1	CD25 interleukin-2Rα expressed on activated T-cells
CACT114A [57]	IgG2b	CD26 dipeptidylpeptidase IV expressed on activated memory T-cells
GS5A [58]	IgG1	CD45R expressed on B-cells, naïve T-cells, and a subset of $CD45R0^+$ memory T-cells
GC44A1 [58]	IgG3	CD45R0 expressed on granulocytes, monocytes, memory T-cells, and γδ T-cells
GB21A [59]	IgG2b	γδ T-cells, δ-chain specific
BAQ44A [60]	IgM	Expressed on B-cells (CD specificity not determined)
DH59B [61]	IgG1	CD172a expressed on monocytes and granulocytes

[a] Unpublished.

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were maintained both short-term and long-term after treatment.

MATERIALS AND METHODS

DNA constructs. The plasmid pSPc5-12 contains a 360-bp *SacI/Bam*HI fragment of the SPc5-12 synthetic promoter [48] in the *SacI/Bam*HI sites of a pSK-GHRH backbone. The synthetic GHRH cDNA, HV-GHRH, was obtained by site-directed mutagenesis of porcine GHRH cDNA (1–40)OH substitutions of Tyr to His at position 1, Ala to Val at position 2, Gly to Ala at position 15, Met to Leu at position 27, and Ser to Asn at position 28 using the Altered Sites II *in Vitro* Mutagenesis System (Promega, Madison, WI, USA) and cloned into the *Bam*HI/*Hin*dIII sites of pSK-GHRH. The GHRH cDNA is followed by the 3′ untranslated region of GH.

Animals. Thirty-two primiparous Holstein cows, 18 to 20 months of age, with an average weight of 547 ± 43 kg, were treated with 2.5 mg pSP-HV-GHRH once during the last trimester of gestation and designated as the treated group. Similarly, 20 pregnant heifers from the same source and of the same breed and age did not receive plasmid treatment and served as controls. Animals calved at age 23 months ± 24 days. Cows were housed in a free-stall barn fitted with fans equipped with water misters for evaporative cooling and exposed to natural daylight. The herd was fed a silage-based total mixed ration *ad libitum* twice daily. Each cow was fitted with a transponder/pedometer that allowed for automatic identification upon entering the stall. At the conclusion of this experiment, all animals treated with plasmid were disposed of in such a manner that their tissues did not enter the food chain. All milk and tissues produced by treated animals were destroyed and did not enter the human food chain. Animal protocols were conducted in accordance with the National Research Council's *Guide for the Care and Use of Laboratory Animals.*

Intramuscular injection of plasmid DNA. The endotoxin-free plasmid (Qiagen, Inc., Chatsworth, CA, USA) preparation of pSPc5-12-HV-GHRH was diluted in water to 5 mg/ml and formulated with poly-L-glutamate 1% wt/wt. Cows were given a total quantity of 2.5 mg pSP-HV-GHRH intramuscularly in the trapezius muscle using a 21-gauge needle (Becton-Dickinson, Franklin Lakes, NJ, USA). Two minutes after injection, the injected muscle was electroporated, 5 pulses, 1 A, 52 ms/pulse, as described [49]. The voltage changes with the change in resistance of the tissue during

TABLE 4: Combinations of mAbs used in three-color FC to determine the composition and frequency of different populations of peripheral blood mononuclear cells and the functional status of CD4 and CD8 αβ T-cells and γδ T-cells

mAb combination	Specificity	Gating strategy used to obtain data on cell subsets
MM1A	CD3	(1) Analyzed ungated to determine the frequency of the $CD3^-CD2^+$ cell population containing NK cells. (2) Analyzed with a gate on $CD3^+$ T-cells to determine the frequency of $CD2^+$ αβ T-cells, $CD2^+$ γδ T-cells, and $CD2^-$ γδ T-cells present in blood.
MUC2A	CD2	
BAT76A	CD2	
GB21A	γδ	
ILA-11A	CD4	Analyzed with a gate on CD4 to determine the frequency of $CD4^+CD45R^+$ naïve and $CD4^+CD45R^+$ memory and $CD45R0^+$ memory T-cells present in peripheral blood.
GS5A	CD45R	
GC44A1	CD45R0	
ILA-11A	CD4	Analyzed with a gate on CD4 to determine the frequency of $CD4^+$ naïve and memory T-cells expressing the activation molecule CD25.
CACT116A	CD25	
GC44A1	CD45R0	
CACT138A	CD4	Analyzed with a gate on CD4 to determine the frequency of $CD4^+$ naïve and memory T-cells expressing the activation molecule CD26.
CACT187A	CD4	
CACT114A	CD26	
GC44A1	CD45R0	
7C2B	CD8	Analyzed with a gate on CD8 to determine the frequency of $CD4^+CD45R^+$ naive and $CD4^+CD45R^+$ memory and $CD45R0^+$ memory T-cells present in peripheral blood.
GS5A	CD45R	
GC44A1	CD45R0	
CACT116A	CD25	Analyzed with a gate on CD8 to determine the frequency of $CD8^+$ naïve and memory T-cells expressing the activation molecule CD25.
GC44A1	CD45R0	
CACT80CA	CD8	Analyzed with a gate on CD8 to determine the frequency of $CD8^+$ naïve and memory T-cells expressing the activation molecule CD26.
BAT82AA	CD8	
CACT114A	CD26	
GC44A1	CD45R0	
GB21A	γδ	Analyzed with a gate on γδ T-cells to determine the frequency of γδ T-cells expressing CD25.
CACT116A	CD25	
GC44A1	CD45R0	
B29A	CD5	Analyzed with no gates to determine the frequency of αβ and γδ $CD5^+$ T-cells, $CD5^+$ and $CD5^-$ B-cells, and monocytes.
BAQ44A	B	
DH59B	CD172a	

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the electroporation (to maintain constant current), and it has been recorded to be between 80 and 120 V/cm. For all injections, 2-cm needles were inserted through the skin into the muscle. Animals were observed immediately after injection and 24 h later for any adverse effects at the electroporation site.

Weight, body condition, and hoof scores. Before treatment, heifers were weighed on the same calibrated scale (Priefert cattle squeeze-chute connected to a Weigh Tronix 915A indicator and WP233 printer; Central City Scale, Central City, NE, USA) and randomly assigned to groups. Two independent dairy animal scientists (Texas A&M University), blinded to the treatment groups, assessed body condition scores prior to treatment, between 60 and 80 DIM, and between 100 and 120 DIM. Cows were scored by both observing and handling the backbone, loin, and rump areas [50], with possible BCS ranging from 1 (very thin cow) to 5 (a severely overconditioned cow). Hoof scores were measured prior to plasmid-GHRH treatment and at 60 DIM. Hoof scores included a 0 (no hoof problem) to 4 (severe hoof problem) evaluation of each foot. Each hoof was assigned an additional 2 points for abscesses eventually present (1 point) and the necessary treatments at any given time (1 point). Possible hoof scores ranged from 0 (no problem) to 24 (severe hoof problems at all 4 feet, abscesses, and intense treatment needed for each hoof).

Complete blood counts and immune markers. Whole blood from all heifers was collected in EDTA and submitted for complete blood count analysis (Texas Veterinary Medical Diagnostic Laboratory, College Station, TX, USA) prior to treatment and at 18 and 300 days posttreatment. Hematology parameters included erythrocyte counts, hematocrit, hemoglobin, total leukocyte count, and differential leukocyte counts (neutrophils, lymphocytes, monocytes, eosinophils, and basophils), platelet count, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, and fibrinogen.

Immune markers were assayed on all treated cattle and controls at days 0 and 18 and on 20 treated and 10 control cows at 300 days posttreatment. Analysis was performed by FC using monoclonal antibodies (mAb) developed in Dr. Davis's laboratory (Department of Veterinary Microbiology/Pathology, CVM, Washington State University, Pullman, WA, USA). Table 3 shows the immunoglobulin isotype and specificity of the mAbs used in the study. Table 4 shows the combinations of mAbs used in three-color FC to determine the composition and frequency of different populations of PBMC in peripheral blood and the functional status of CD4 and CD8 $\alpha\beta$ T-cells and $\gamma\delta$ T-cells. As shown, two mAbs of the same specificity have been used in some combinations to increase the intensity of the fluorescent signal on CD4 and CD8 T cells. Ten milliliters of blood was obtained at the times indicated and processed for FC [51]. The blood was lysed in Tris-buffered NH_4Cl, washed in phosphate-buffered saline containing acid citrate dextrose (PBS/ACD), and then distributed in 96-well conical bottom tissue culture plates containing the different combinations of mAbs. The cells were incubated for 15 min on ice, washed 3× in PBS/ACD, and incubated another 15 min with different combinations of isotype-specific goat anti-mouse antibodies conjugated with fluorescein, phycoerythrin, or phycoerythrin-Cy5 (Southern Biotechnology Associates, Birmingham, AL, USA; Caltag Laboratories, Burlingame, CA, USA). The cells were then washed 2× and fixed in PBS-buffered 2% formaldehyde. The cells were kept in the refrigerator until examined on a Becton–Dickinson FACSort equipped with CellQuest software. Data were analyzed on Flow Jo (Tree Star, Inc., San Carlos, CA, USA) and FCS Express (De Novo Software, Thornton, ON, Canada) software. Unless otherwise stated, data are presented as percentages of the total population assayed with a particular monoclonal antibody or combination thereof (e.g., total $CD4^+$ and $CD4^-$ cells represent 100% of cells assayed; total $CD2^-CD3^-$, $CD2^-CD3^+$, $CD2^+CD3^-$, or $CD2^+CD3^+$ represent 100% of cells assayed with both CD2 and CD3 antibody, etc.).

Biochemistry and insulin measurements. Serum samples were collected at 60 and 100 DIM. Serum was aliquoted for radioimmunoassay and biochemical analysis and stored at −80°C prior to analysis. Biochemical analysis occurred within 48 h after serum collection (Texas Veterinary Medical Diagnostic Laboratory). Serum biochemical endpoints included alanine aminotransferase, γ-glutamyltransferase, creatine phosphokinase, total bilirubin, total protein, albumin, globulin, blood urea nitrogen, creatinine, phosphorus, calcium, and glucose. Insulin and IGF-I assays were performed within 90 days after serum collection. Samples were analyzed for glucose and insulin levels by an independent laboratory (Texas Veterinary Medical Diagnostic Laboratory). All samples were analyzed in the same assay. The assay variability was 3.6% for the insulin assay and 4.4% for the glucose assay. Total proteins were measured using a Bio-Rad protein assay kit on the serum samples (Bio-Rad Laboratories, Hercules, CA, USA).

Radioimmunoassay for IGF-I. Serum IGF-I was measured using a heterologous human immunoradiometric assay kit following the manufacturer's protocol (Diagnostic System Labs, Webster, TX, USA). The kit employs an extraction step to remove binding protein interference. All samples were run in the same assay. The intra-assay variability was 4%. Cross-reactivity of human IGF-I antibody for bovine IGF-I is 100%.

Statistical analysis. Data consisted of repeated measures at different time points with unequal allocation of experimental units to treatment groups (treated $n = 32$, controls $n = 20$). Additional comparisons were performed when a significant ($P < 0.05$) treatment × day interaction was detected. A mixed model using SAS (analysis of simple main effects) was used to examine if there were any significant differences among the groups of each variable at different time points. Categorical data, such as culling rate and hoof problems, were analyzed by ANOVA. Data were coded with numerical values such that ANOVA could be performed. The total hoof score for each animal was used in the analysis of this parameter. For mortality rates, we developed an equivalent scoring system: alive = 1, dead = 0. Serum IGF-I was analyzed by ANOVA for repeated measures. Values compared with Student's t test, ANOVA, or linear regression are presented under Results, with $P < 0.05$ taken as the level of statistical significance.

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EXHIBIT B

A Dictionary of GENETICS

Sixth Edition

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ROBERT C. KING
Emeritus Professor, Northwestern University

WILLIAM D. STANSFIELD
Emeritus Professor, California Polytechnic State University

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interspersed elements *See* repetitious DNA.

interstitial cells cells that lie between the testis tubules of vertebrates and secrete testosterone.

intervening sequence *See* intron.

intrachromosomal aberration *See* translocation.

intrachromosomal recombination sister chromatid exchange (*q.v.*).

intrachromosomal translocation *See* translocation.

intragenic complementation *See* interallelic complementation.

intragenic recombination recombination between mutons of a cistron. Such recombination is characterized by negative interference and by nonreciprocality (recovery of either wild-type or double-mutant recombinants, but not both from the same tetrad).

intragenic suppression *See* suppression.

intrasexual selection *See* sexual selection.

introgression *See* introgressive hybridization.

introgressive hybridization the incorporation of genes of one species into the gene pool of another. If the ranges of two species overlap and fertile hybrids are produced, they tend to backcross with the more abundant species. This process results in a population of individuals, most of which resemble the more abundant parents but that also possess some characters of the other parent species. Local habitat modification can lead to mixing of previously distinct gene pools. Introduced species (or subspecies) can generate extinction of the older species by hybridization and introgression. The use of molecular markers has greatly increased the ability to detect and quantify interspecific gene exchanges. For example, phylogenetic trees based on chDNAs have shown many examples of both recent and ancient exchanges of chloroplasts between sympatric species. *See* chloroplast DNA (chDNA), wolf.

intromittent organ any male copulatory organ that implants sperm within the female.

intron in split genes (*q.v.*), a segment that is transcribed into nuclear RNA, but is subsequently removed from within the transcript and rapidly degraded. Most genes in the nuclei of eukaryotes contain introns and so do mitochondrial genes and some chloroplast genes. The number of introns per gene varies greatly, from one in the case of rRNA genes to more than 30 in the case of the yolk protein genes of *Xenopus*. Introns range in size from less than 100 to more than 10,000 nucleotides. There is little sequence homology among introns, but there are a few nucleotides at each end that are nearly the same in all introns. These boundary sequences participate in excision and splicing reactions. The first introns of some genes have been shown to contain tissue-specific enhancers. *See* Appendix C, 1977, Roberts and Sharp; 1978, Gilbert; 1983, Gillies *et al.*; alternative splicing, *Caenorhabditis elegans*, enhancers, exon, posttranscriptional processing, R-loop mapping, splice junctions, spliceosome, transcription unit.

intron intrusion the disruption of a preexisting gene by the insertion of an intron into a functional gene. Intron intrusion and the exon shuffling (*q.v.*) along with junctional sliding (*q.v.*), have been proposed as mechanisms for evolutionary diversification of genes.

intron-mediated recombination *See* exon shuffling.

intron origins two conflicting hypotheses have been invented to explain the origin of introns. The *introns early hypothesis* assumes that the DNA molecules in which genes originated initially contained random sequences of nucleotides. The random distribution of stop codons permitted only short reading frames to accumulate. Next, a mechanism arose that allowed splicing out regions containing stop codons from the primary message, and so proteins of greater length and with more useful biochemical functions could be translated and selected. The original short reading frames became the exons of present-day genes, while the introns represent segments containing splice junctions originally designed to remove deleterious stop signals. The *introns late hypothesis* assumes that genes arose from short reading frames that grew larger by duplications and fusions. Introns arose secondarily as a result of insertions of foreign DNA into these genes. Thus, present-day introns are the descendants of ancient transposons (*q.v.*).

intussusception **1.** the growth of an organism by the conversion of nutrients into protoplasm. **2.** the deposition of material between the microfibrils of a plant cell wall. **3.** the increase in surface area of the plasmalemma by intercalation of new molecules between the existing molecules of the extending membrane.

in utero within the uterus.

inv *See* symbols used in human cytogenetics.

in vacuo in a vacuum.

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